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Clinical Evaluation of a New Digitoxin Enzyme-Immunoassay

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Summary: A solid-phase enzyme-immunoassay for the determination of the digitoxin concentration in human serum (Enzymun-Test® Digitoxin) was developed, and subsequently evaluated in seven laboratories. The test is based on the competition principle. Polystyrene tubes coated with anti-digitoxin antibodies (from sheep) were used as the solid phase. In the concentration range 10–40 µg/l, the coefficient of variation for the majority of laboratories was between 2 and 7%. The day-to-day precision only slightly differed from the within-run precision. The measuring range was between 4 and 60 µg/l. Enzymun-Test® Digitoxin showed good agreement with three known methods for digitoxin determination. No influence on the values was observed in lipaemic, uraemic and icteric samples, dysproteinaemia sera and in the presence of various digoxin derivatives. The new enzyme-immunoassay permits the practical and reliable determination of serum digitoxin and is suited for use in routine analysis.

Klinische Evaluierung eines neuen Digitoxin-Enzym-Immunoassay

Zusammenfassung: Zur Bestimmung der Digitoxin-Konzentration im Humanserum wurde ein Festphasen-Enzymimmunoassay (Enzymun-Test® Digitoxin) entwickelt und in sieben Laboratorien evaluiert. Der Test beruht auf dem Kompetitionsprinzip. Als Festphase werden mit Anti-Digitoxin-Antikörpern (Schaf) beschichtete Polystyrolröhrchen eingesetzt. Im Konzentrationsbereich von 10 bis 40 µg/l lagen die Variationskoeffizienten der meisten Labors zwischen 2 und 7%. Die Präzision von Tag zu Tag unterschied sich nur unwesentlich von der innerhalb einer Serie. Der Meßbereich erstreckte sich von 4 bis 60 µg/l. Enzymun-Test® Digitoxin zeigte eine gute Übereinstimmung mit drei bekannten Methoden zur Digitoxin-Bestimmung. In lipämischen, urämischen und ikterischen Proben, Dysproteinämie-Seren sowie bei verschiedenen Digoxin-Derivaten wurde keine Beeinflussung der Werte beobachtet. Dieser neue Enzymimmunoassay gestattet eine sichere und praktikable Bestimmung des Digitoxins im Serum und ist für den Einsatz in der Routine geeignet.

Introduction

Provided that suitable sampling times and sampling conditions are used, the determination of glycoside concentrations in human serum provides useful information on the actual intake, the distribution and the excretion of *Digitalis* glycosides. Digitoxin determinations help on the one hand to avoid dangerous long term effects resulting from the comparatively long half-life of digitoxin (1, 2), and on the other hand enable an improved assessment of severe intoxications (3, 4).

Measurement of serum glycoside concentrations yields only a limited amount of information, however, on the myocardial uptake of the glycoside and the degree of receptor-binding of the glycoside and its active metabolites, which in the final reckoning is responsible for the action of digitalis. The validity of the information obtained from measurements of the glycoside concentration can be further restricted by additional unexpected and possibly rapidly changing pathological conditions, e. g. renal failure, thyroid gland dysfunctions and derangement of the electrolyte and water balance (3–6).

In addition to the already known immunoassays for determining digitoxin in serum (6–9), a new enzyme-immunoassay has recently been presented (10). The investigation of this test (Enzymun-Text® Digitoxin) in 7 laboratories is described here.

Methods and Materials

Test principle and test procedure for Enzymun-Test® Digitoxin[1])

Enzymun-Test® Digitoxin is an enzyme-immunosassay based on the competition principle. The digitoxin from the serum sample (20 µl) and the digitoxin conjugate (1 ml, digitoxin coupled with peroxidase from horse-radish) compete for digitoxin antibodies attached to the inner wall of polystyrene tubes. After a 30 min period of incubation at room temperature, the non-bound digitoxin conjugate is removed by aspirating the liquid followed by a washing step. A solution of diammonium 2,2′-azino-bis-(3-ethyl-benzothioazoline-6-sulphonate) (ABTS®) is then added, and after 30 min the coloration resulting from the wall-bound peroxidase activity is measured spectrometrically at 405 nm. The intensity of colour produced is inversely proportional to the digitoxin concentration. Evaluation is carried out via a hyperbolic calibration curve prepared with the aid of 5 standards. The composition of the reagents is listed in table 1.

Tab. 1. Composition of the reagents used in Enzymun-Test® Digitoxin (manufacturer's information).

1.	Binding reaction	
	Buffer	
	Phosphate buffer, pH = 6.8	40 mmol/l
	Bovine serum albumin	2.5 g/l
	Coated tubes	
	Wall-bound polyclonal digitoxin antibodies from sheep serum, specificity: see table 4	
	Digitoxin-peroxidase conjugate	
	Peroxidase	approx. 30 U/l
	Bovine serum albumin	
2.	Indicator reaction	
	Phosphate/citrate buffer, pH = 4.4	100 mmol/l
	H_2O_2 (sodium perborate)	3.2 mmol/l
	ABTS®	1.9 mmol/l
3.	Standards	
	Digitoxin added to digitoxin-free human serum, lyophilised; concentrations: 0 µg/l, 7.5 µg/l, 15 µg/l, 30 µg/l, 60 µg/l	
4.	Control serum	
	Digitoxin added to digitoxin-free human serum, lyophilised	

Sample material and quality control

Human sera from normal donors and patients receiving digitoxin therapy were used for the investigations; fresh sera were used for investigating within-series precision, linearity and interference, whereas deep-frozen sera were used for investigating day-to-day precision and carrying out method comparisons. The control serum contained in the Enzymun-Test® Digitoxin pack was also analysed for quality control purposes. In the method comparisons, the control sera recommended by the respective manufacturers were used.

[1]) Commercially available:
Enzymun-Test® Digitoxin
Cat. No.: 759 333
Company: Boehringer Mannheim GmbH

Participants, reagents and equipment

The laboratories taking part in the study are designated with the letters A to G. Results from the Boehringer Mannheim laboratories have been given the letter H. The order of appearance of the authors' names on the title page of this paper does not correspond to the sequence A to G.

Table 2 provides an overview of the comparison methods, equipment and test variants.

Tab. 2. Participants and their methods.

Laboratory	Working instructions for Enzymun-Test® Digitoxin			Comparison methods*
	manual		ES 22	
	without	with stop reagent		
A		×	×	1
B		×	×	1, 3
C	×			1, 2
D		×	×	2
E			×	2, 3
F			×	3
G			×	2
H	×	×	×	1, 2

*1: RIA 1
2: RIA 2
3: Fluorescence polarisation immunoassay

For the method comparisons, two radioimmunoassays and a fluorescence polarisation immunoassay were used:

Coat-A-Count, ^{125}I-Digitoxin (Diagnostic Products, Corp., Los Angeles, Cal., USA), GammaCoat™, ^{125}I-Digitoxin (Clinical Assays, Cambridge, Mass., USA) and Abbott TDx-Digitoxin (Abbott Laboratories, North Chicago, Il., USA).

With the exception of one laboratory, all of the participants used Enzymun-Test® Digitoxin in association with the Enzymun-Test® System ES 22 (Boehringer Mannheim GmbH), hereafter referred to as ES 22. By combining a spectrometer (Photometer 4010), computer (Epson HX-20 microcomputer/Interface 4010) and a pipetting/wash station, ES 22 enables the test to be largely mechanised. Curve fitting is achieved by a cubic spline interpolation (15).

For the manual technique, four laboratories tested a reagent, which can be used to stop the development of colour in the indicator reaction of Enzymun-Test® Digitoxin (Enzymun-Test® Stop Reagent, Cat. No.: 811 769, Boehringer Mannheim GmbH).

This reagent contains catalase (= 150 kU/l) in an acetate buffer (50 mmol/l, pH 5.5) and 5 g/l detergent. By adding 0.1 ml of the stop reagent to 1 ml of substrate buffer solution, the oxidation of the chromogen ABTS® is interrupted, while at the same time the coloured ABTS® radical is stabilised.

Evaluation protocol

1. Within-series (20 duplicate determinations) and between-day (single values from 10 days) precision of Enzymun-Test® Digitoxin was measured in the concentration range from 10–40 µg/l.

2. In order to ascertain the lower detection limit, the zero standard was measured 30 times manually (without stop reagent) and with the ES 22. In accordance with the definition by *Kaiser* (11), the lower detection limit was calculated from the difference in measured signals between the mean and 3 standard deviations. The results obtained by that procedure were checked by measuring specimens having digitoxin concentrations near the lower detection limit (n = 20, digitoxin standards: 2 µg/l and 4 µg/l).

3. Within the scope of a collaborative study, all of the participants determined three "unknown" human serum-based, lyophilized specimens containing different digitoxin concentrations (duplicate measurements on different 10 days).

4. The specificity of the digitoxin antibody was checked with the digitoxin-like compounds listed in table 4. The interference of 43 commonly used drugs was investigated in vitro using twice the toxic concentrations according to l. c. (12). Lipaemic, uraemic and icteric (up to 171 µmol/l bilirubin) sera were included in the method comparison studies with the reagents named above.

   In order to check the possibility of interference by haemolysis, human serum containing 14 µg/l digitoxin was spiked stepwise with haemolysate up to a final concentration of 20 g/l Hb.

   The influence of various human sera with pathologically altered protein concentrations (see tab. 3) was investigated by a stepwise blending with a human serum having a high digitoxin concentration.

Tab. 3. Characteristics of human sera from four patients with dysproteinaemia. These sera were used to investigate the influence of dysproteinaemia on Enzymun-Test® Digitoxin (Laboratory E).

Patient	IgA (g/l)	IgM (g/l)	IgG (g/l)	Kappa/ Lambda
1	15.30	1.23	19.70	–
2	0.85	92.00	8.02	7.90
3	3.19	39.20	6.02	2.40
4	0.42	0.65	35.90	0.02

5. Method comparison studies in human sera were made to investigate the degree of agreement between various test versions of Enzymun-Test® Digitoxin (manual procedure with/without stop reagent; ES 22-procedure).

6. The accuracy of Enzymun-Test® Digitoxin was investigated in the following experiments:

   – Recovery of digitoxin in a (digitoxin-free) human serum after spiking with definite amounts of the 60 µg/l digitoxin standard. Recovery was checked by visual inspection of the graphic presentation obtained by plotting found concentrations as a function of dilution.

   – Comparison studies with human sera covering a wide range of concentrations with three other frequently used tests (tab. 2).

   Statistical evaluation was performed according to the standardised principle component analysis (13, 14).

Results and Discussion

Precision

Depending upon the concentration, the majority of coefficients of variation were between 2 and 7% with the day-to-day scatter being only slightly higher than within series (figs. 1 and 2). The test system hence shows good precision in the central concentration range from about 10 to 40 µg/l.

When the timing cycles are carefully observed, the values measured manually show precision similar to that obtained with the ES 22. The procedure is simplified by the stop reagent, which removes the need for careful timing of spectrometric measurements.

Analytical range limits

In the determination of the lower detection limit values between 1 and 2 µg/l were obtained, with values down to 1 µg/l using the ES 22. Imprecision studies in that concentration range yielded coefficients of variation of 39% (2 µg/l) and 9% (4 µg/l). Thus the diagnostically useful measuring range extends from 4 µg/l to 60 µg/l (highest standard) digitoxin.

Collaborative study

The results of the collaborative, interlaboratory trial (tab. 4) show interlaboratory coefficients of variation of 4.6%, 5.6% and 6.9% for the three samples used. These data document a good comparability of values from one laboratory to another.

Specificity

The results shown in table 5 show that the wall-bound digitoxin antibodies are highly specific (laboratory H): The metabolites of digitoxin showed cross-reactions of 80 to 100%, whereas all of the digoxin derivatives showed cross-reactions of less than 2%. Cross-reactions of 53% and 41% respectively were shown by the alpha- and beta-forms of

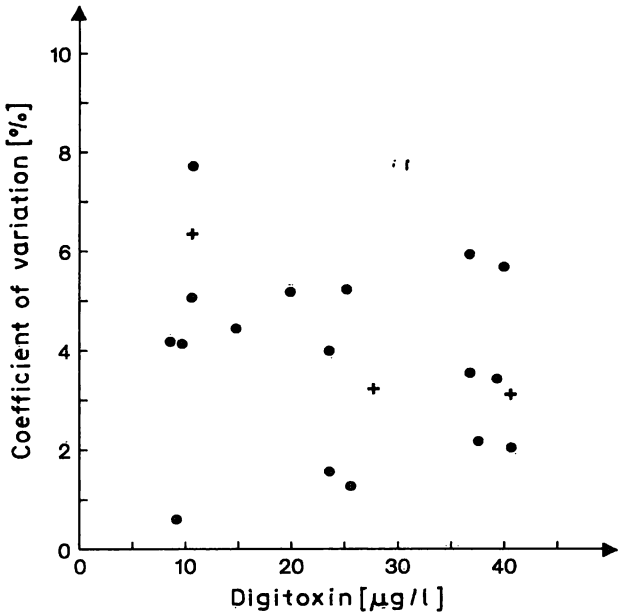

Fig. 1. Within-series precision; groups of 20 duplicate determinations in human sera having differing digitoxin concentrations
+ manual procedure
● ES 22

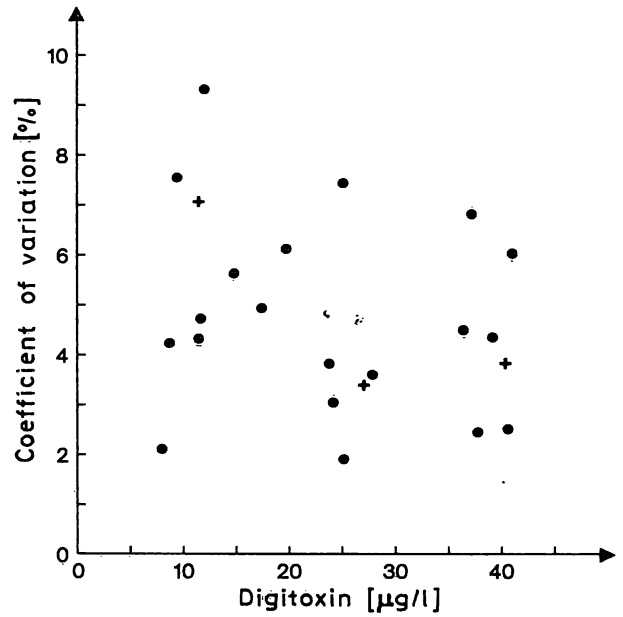

Fig. 2. Day-to-day precision; single values obtained on 10 days from portions of frozen human serum with differing digitoxin concentrations
+ manual procedure
● ES 22

Tab. 4. Results of the collaborative study; duplicate determinations carried out on 10 days (manual in 2 laboratories; with ES 22 in 6 laboratories)
*Lyophilised human serum spiked with digitoxin (approx. 10, 20, 40 µg/l)

Sample	Target value (µg/l)	Mean value (µg/l)	Median (µg/l)	Extreme values (µg/l)	Inter-laboratory coefficient of variation (%), (n = 80)
1*	–	11.7	11.7	9.9–14.6	6.9
2* (control serum)	20.6	20.5	20.4	18.5–23.5	4.6
3*	–	41.0	40.9	36.7–46.2	5.6

K-strophanthin. Since the simultaneous use of strophanthin (intravenous) and digitoxin is unusual and illogical, this high cross-reaction should not be seen as a disadvantage.

Tab. 5. Cross reactions, calculated on a weight basis (Laboratory H).
% Cross reaction:

$$\frac{\text{50\% intercept analyte} \times 100}{\text{50\% intercept cross-reacting substance}}$$

Analyte	Concentration of the cross-reacting substance (µg/l) for 50% intercept	Percent cross reaction
Digoxin	1000	1.5
β-Methyldigoxin	1150	1.3
α-Acetyldigoxin	1100	1.3
β-Acetyldigoxin	1275	1.2
Lanatoside C	1600	1.0
Digoxigenin	1050	1.5
Digoxigenin-mono-digitoxoside	850	1.8
Digoxigenin-bis-digitoxoside	1050	1.5
Digitoxigenin	90	86.8
Digitoxigenin-mono-digitoxoside	15	98.7
Digitoxigenin-bis-digitoxoside	18.5	80.0
Prednisolone	100000	0.02
Spironolactone	100000	0.02
Canrenone	55000	0.03
Prednisone	100000	0.02
Progesterone	100000	0.02
Dihydrodigoxin	77000	0.02
Proscillaridin	470	3.1
k-Strophantin-β	36	41.1
k-Strophantin-α	28	52.9
Cortisol	100000	0.02
g-Strophantin (Ouabain)	2500	0.6

Accuracy

The results obtained in the recovery experiment (fig. 3) show an excellent recovery of digitoxin in serum. As can be seen in table 6, values for the test versions of Enzymun-Test® Digitoxin, carried out on a manual or partially automated basis, are in close agreement.

Results of the same quality were found in comparisons with test kits of other manufacturers (tab. 7).

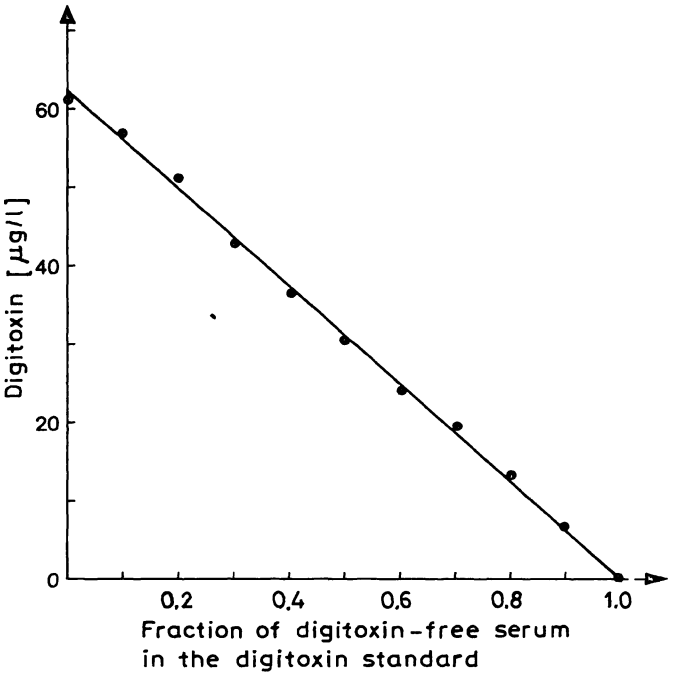

Fig. 3. Recovery of digitoxin in human serum (Laboratory D). The digitoxin standard contained 60 µg/l

Tab. 6. Comparison of the three working procedures/applications of Enzymun-Test® Digitoxin

Working specification x	Working specification y	Range tested (µg/l)	Regression equation	r	Paired values	Median of the relative differences (%)	Laboratory
Manual	ES 22	5–60	$y = -2.108 + 1.049x$	0.944	102	−4.6	H
Manual	manual/with stop reagent	5–60	$y = 0.378 + 0.921x$	0.908	102	−8.6	H
Manual/stop reagent	ES 22	2–60	$y = -1.658 + 1.075x$	0.960	70	−2.9	A
Manual/stop reagent	ES 22	8–65	$y = 0.055 + 0.945x$	0.985	50	−5.4	D

Tab. 7. Comparison of Enzymun-Test® Digitoxin with other methods

Test x	Test y Enzymun-Test® Digitoxin	Range tested (µg/l)	Regression equation	r	Paired values	Median of relative differences (%)	Laboratory
RIA 1	ES 22	7–55	$y = -1.545 + 1.155x$	0.900	70	7.8	A
RIA 1	manual	5–40	$y = -0.413 + 1.185x$	0.956	45	14.1	C
RIA 1	manual	5–60	$y = 2.078 + 0.921x$	0.911	102	− 0.1	H
RIA 2	ES 22	4–70	$y = 3.255 + 0.908x$	0.919	55	2.4	E
RIA 2	ES 22	5–65	$y = -0.747 + 0.991x$	0.893	102	− 6.3	H
Fluorescence polarisation immunoassay	ES 22	5–60	$y = 0.295 + 1.049x$	0.976	47	3.3	B

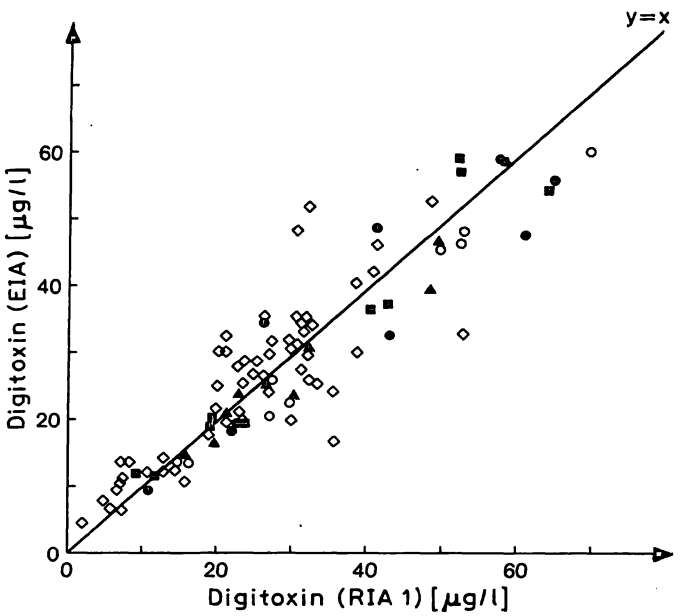

Fig. 4. Comparison of Enzymun-Test® Digitoxin (y) with RIA 1 (x) in 102 human sera (Laboratory H, statistical data in table 6)
▲: icteric
□: no information
●: lipaemic
○: uraemic
■: dysproteinaemia

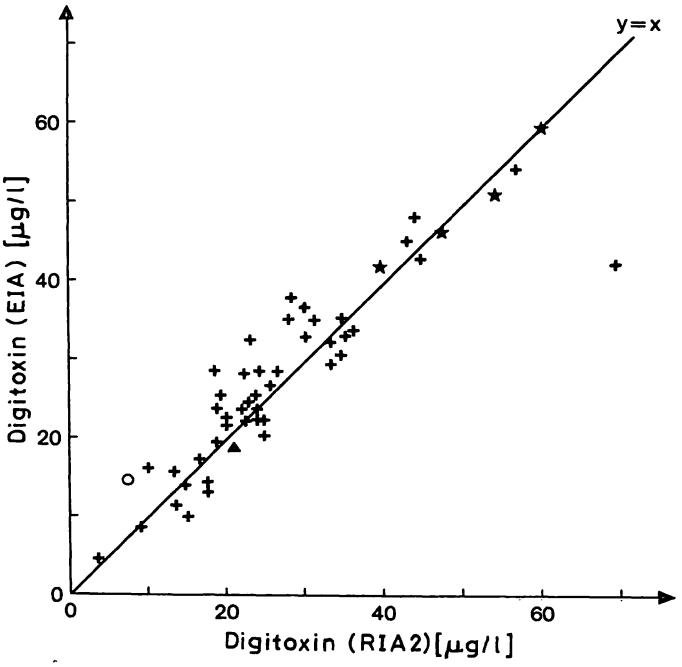

Fig. 5. Comparison of Enzymun-Test® Digitoxin (y) with RIA 2 (x) in 55 human sera (Laboratory E, statistical data in table 6)
▲: uraemic samples
+: no information
*: haemolytic samples
○: containing digoxin

Two typical plots are shown in figures 4 and 5. As part of the method comparisons, the use of plasma was also investigated (laboratories C, E, F and G). It was found here that irrespective of the anticoagulants used, the values measured for digitoxin were about 10–20% higher than with serum; for this reason only serum is recommended as sample material.

Since a reference method for the measurement of digitoxin in serum is not available, the accuracy of this new enzyme-immunoassay could only be assessed by recovery experiments and comparison studies with established methods. These investigations indicate that Enzymun-Test® Digitoxin exhibits the same accuracy as accepted radioimmunoassays.

Interfering substances

No indications of possible interfering factors were found in the method comparison studies in lipaemic, uraemic and icteric (up to 171 µmol/l bilirubin) sera (figs. 4 and 5).

In the drug interference studies all recoveries were between 90 and 110% in terms of the initial value (tab. 8). The pharmaceuticals shown in table 8 should not therefore lead to any method-mediated influence on the values measured with Enzymun-Test® Digitoxin.

Haemoglobin did not interfere up to the highest concentration tested (20 g/l, see fig. 6).

No interferences were observed in the investigations of four human sera containing pathologically altered protein concentrations. The results obtained with the serum from patient 2 (see tab. 3) are shown in figure 7.

Conclusions

These studies show that Enzymun-Test® Digitoxin produces values for digitoxin that are largely free from interference and which are in good agreement with the results from other commercially available tests. This test is an alternative to radioimmunological methods, and enables the reliable and simple determination of digitoxin, also in smaller routine laboratories.

Acknowledgement

We would like to thank Mr. *McRoberts* (Boehringer Mannheim GmbH) for translating the original German manuscript into English.

Tab. 8. Influence of pharmaceuticals, used at twice the toxic concentration (12).
Duplicate determinations using the ES 22 (Laboratory H).

Pharmaceuticals	Recovery of digitoxin (%)
Acetylsalicylic acid	97.3
Ampicillin	97.1
Meglium salt of amidotrizoic acid	100.8
Ascorbic acid	99.7
Meglium salt of iodipamide	92.7
Glibenclamide	101.7
Carbochromen	102.6
Quinidine (bisulphate)	100.5
Chloramphenicol	93.0
Chlordiazepoxide	93.0
Bezafibrate	99.3
Caffeine	104.5
Dextran (polyglucose)	97.9
Etnaverine	96.5
Furosemide	91.3
Gelatine	90.1
Indometacin	99.9
Methaqualone	97.6
Methyldopa	102.4
Nicotinic acid	102.0
Nitrofurantoin	96.0
Noramidopyrine-methane-sulphonic acid	96.8
Oxazepam	106.5
Oxyphenbutazone	105.9
Oxytetracycline	110.0
Paracetamol	103.7
Phenazopyridine	104.4
Phenobarbitone	104.4
Phenprocoumon	101.1
Phenytoin	108.1
Probenecid	106.8
Procaine	103.3
Pyridamol	108.1
Pyritinol	100.5
Sulphamethoxazole	106.5
Theophylline	107.3
Trimethoprim	102.8
Carbimazole	95.2
Methylthiouracil	103.6
Allopurinol	101.6
Bilirubin	100.3
Methotrexate	100.6
Dexamethasone	105.3

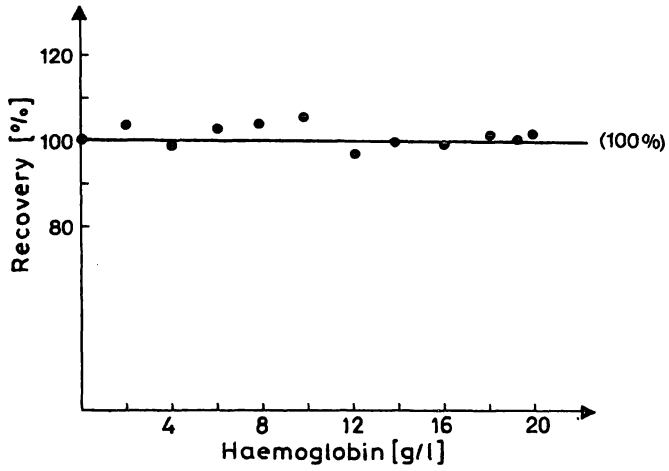

Fig. 6. Influence of haemolysis on Enzymun-Test® Digitoxin carried out using the ES 22 (digitoxin content 14 µg/l)

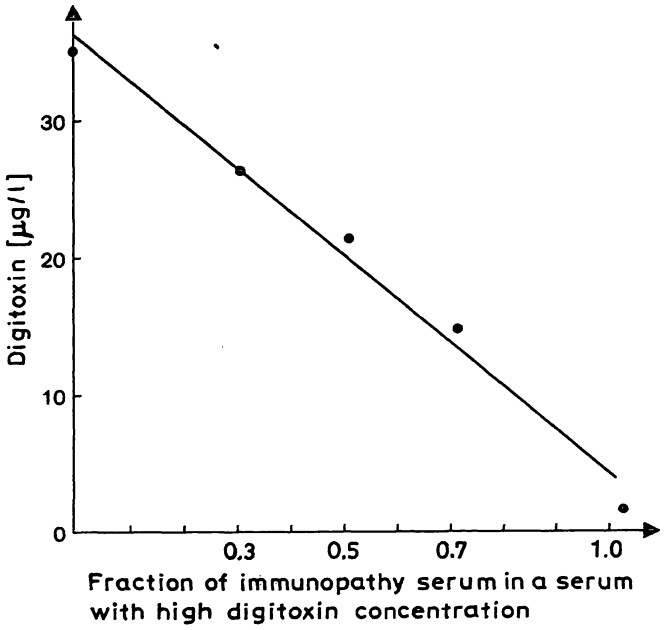

Fig. 7. Influence of a monoclonal immunopathy serum (patient 2 in table 3) on the linearity of Enzymun-Test® Digitoxin (Laboratory E)

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